Supporting Information

Probing the Catalytic Charge-Relay System in Alanine Racemase with Genetically Encoded Histidine Mimetics

Vangmayee Sharma, Yane-Shih Wang, and Wenshe Liu

Contents

1. WT alanine racemase amino acid sequence from *Bacillus stearothermophilus*:

MNDFHRDTWA EVDLDAIYDN VANLRRLLPD DTHIMAVVKA NAYGHGDVQV ARTALEAGAS
RLAVAFLDEA LALREKGIEA PILVLGASRP ADAALAAQQR IALTVFRSDW LEEASALYSG
PFPIHFHLKM DTGMGRLGVK DEEETKRIVA LIERHPHFVL EGVYTHFATA DEVNTDYFSY
QYTRFLHMLE WLPSRPPLVH CANSAASLRF PDRTFNMVRF GIAMYGLAPS PGIKPLLPYP
LKEAFSLHSR LVHVKKLQPG EKVSYGATYT AQTEEWIGTI PIGYADGWLR RLQHFHVLVD
GQKAPIVGRI CMDQCMIRLP GPLPVGTKVT LIGRQGDEVI SIDDVARHLE TINYEVPCTI
SYRVPRIFFR HKRIMEVRNA IGRGESSALE HHHHHH

H127
H166
H200

Highlighted amino acids indicate the residues replaced with Mh or Ta to obtain alanine racemase mutants.

2. Nucleotide sequence of WT alanine racemase from *Bacillus stearothermophilus*:

ATGAACGACTTTCATCGCGATACGTGGGCGGAAGTGGATTTGGACGCCATTTACGACAATGTGGCGAATTTG
CGCCGTTTGCTGCCGGACGACACGCACATTATGGCGGTCGTGAAGGCGAACGCCTATGGACATGGGGATGT
GCAGGTGGCAAGGACAGCGCTCGAAGCGGGGGCCTCCCGCCTGGCGGTTGCCTTTTTGGATGAGGCGCTCG
CTTTAAGGGAAAAAGGAATCGAAGCGCCGATTCTAGTTCTCGGGGCTTCCCGTCCAGCTGATGCGGCGCTGG
CCGCCCAGCAGCGCATTGCCCTGACCGTGTTCCGCTCCGACTGGTTGGAAGAAGCGTCCGCCCTTTACAGCG
GCCCTTTTCCTATTCATTTCCATTTGAAAATGGACACCGGCATGGGACGGCTTGGAGTGAAAGACGAGGAAG
AGACGAAACGAATCGTAGCGCTGATTGAGCGCCATCCGCATTTTGTGCTTGAAGGGGTGTACACGCATTTTG
CGACTGCGGATGAGGTGAACACCGATTATTTTTCCTATCAGTATACCCGTTTTTTGCACATGCTCGAATGGCT
GCCGTCGCGCCCGCCGCTCGTCCATTGCGCCAACAGCGCAGCGTCGCTCCGTTTCCCTGACCGGACGTTCAA
TATGGTCCGCTTCGGCATTGCCATGTATGGGCTTGCCCCGTCGCCCGGCATCAAGCCGCTGCTGCCGTATCC
ATTAAAAGAAGCATTTTCGCTCCATAGCCGCCTCGTACACGTCAAAAAACTGCAACCAGGCGAAAAGGTGA
GCTATGGTGCGACGTACACTGCGCAGACGGAGGAGTGGATCGGGACGATTCCGATCGGCTATGCGGACGGC
TGGCTCCGCCGCCTGCAGCACTTTCATGTCCTTGTTGACGGACAAAAGGCGCCGATTGTCGGCCGCATTTGC
ATGGACCAGTGCATGATCCGCCTGCCTGGTCCGCTGCCGGTCGGCACGAAGGTGACACTGATTGGTCGCCAA
GGGGACGAGGTAATTTCCATTGATGATGTCGCTCGCCATTTGGAAACGATCAACTACGAAGTGCCTTGCACG
ATCAGTTATCGAGTGCCCCGTATTTTTTTTCGCCATAAGCGTATAATGGAAGTGAGAAACGCCATTGGCCGC
GGGGAAAGCAGTGCACTCGAGCACCACCACCACCACCACTGA

3. Construction of Plasmids:

The AlaR gene was amplified from pET23a-wtAlaR by flanking primers, AlaR- KpnI-F and AlaR- NcoI-R. The gene product was digested by *KpnI* and *NcoI*, purified and ligated into pBad vector digested by *KpnI* and *NcoI* to generate plasmid pBAD-AlaRwt-His6x as shown in **Figure S1 (B)**. The *NcoI* restriction site was then removed from the pBAD vector by primers Del-pBAD-alaR-F and Del-pBAD-alaR-R.

The plasmids pBAD-AlaRH166-His6x, pBAD-AlaRH200-His6x and pBAD-AlaRH127-His6x were all derived from the plasmid pBAD-AlaRwt-His6x by introducing TAG mutations by site-directed mutagenesis using the following primers respectively: (i) His 166-QC-F and His 166-QC-R, (ii) His 200-QC-F and His 200-QC-R, (iii) His 127-QC-F and His 127-QC-R.

All the plasmid sequences were confirmed by DNA sequencing. All oligonucleotide primers were purchased from Integrated DNA Technologies, Inc.

4. Primer List:

AlaR- KpnI-F, 5'-GCC GGT GGT ACC TCA GTG GTG GTG-3'
AlaR- NcoI-R, 5'-GCC TGG CCA TGG ATG AAC GAC TTT CAT C-3'

Del-pBAD-alaR-F, 5'-ATG AAC GAC TTT CAT CGC GAT ACG TGG GCG-3'
Del-pBAD-alaR-R, 5'-GGT TAA TTC CTC CTG TTA GCC CAA AAA ACG GG-3'

His 166-QC-F, 5'-TTT GCG ACT GCG GAT GAG GTG AAC AC-3'
His 166-QC-R, 5'-CTA CGT GTA CAC CCC TTC AAG CAC AA-3'

His 200-QC-F, 5'-TGC GCC AAC AGC GCA GCG TCG CTC CG-3'
His 200-QC-R, 5'-CTA GAC GAG CGG CGG GCG CGA CGG CAG-3'

His 127-QC-F, 5'-TTG AAA ATG GAC ACC GGC ATG GGA CG-3'
His 127-QC-R, 5'-CTA GAA ATG AAT AGG AAA AGG GCC GCT G-3'

5. Expression of alanine racemase and mutants:

For expression of alanine racemase mutant proteins, *e. coli* Top10 cells were co-transformed with a pEVOL-pylT-PylRS(N346A/C348A) plasmid containing $tRNA^{Pyl}_{CUA}$ and two copies of PylRS N346A/C348A mutant[1] and corresponding pBAD-alaRHxxx-His6x plasmid with TAG mutation at the required histidine position. The transformed cells were grown overnight at 37°C, 250 rpm, in auto-induction media (with tyrosine, cysteine, histidine, phenylalanine and tryptophan left out of the media)[2] and supplemented with 2 mM of the NCAA. The culture was inoculated in the auto-induction media (50 ml) containing 100 mg/mL ampicillin and 34 mg/mL chloramphenicol and Mh or Ta were added to final concentration of 2mM after growing the cells in the media for 30 min. The protein expression was induced when the culture reached OD_{600} of 0.5 by adding arabinose to a final concentration of 0.2 %. After 24h induction, cells were harvested, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with 20 µM pyridoxal-5'-phosphate (PLP) and sonicated. The cell lysate was clarified by centrifugation (30 min, 11,000 g, 4ºC). The supernatant was injected into a 30 mL Ni^{2+}-NTA column (Qiagen) and washed with 60 to 90 mL lysis buffer. Protein was finally eluted out by running an imidazole gradient from 10 mM to 250 mM in lysis buffer. Pure fractions were collected and concentrated. The eluted protein was dialyzed against 1X PBS buffer with 20 µM PLP concentrated using an Amicon Ultra-15 Centrifugal Filter (10,000 MWCO). The purified proteins were analysed by 12% SDS-PAGE. AlaRHxxx-His6X mutant proteins incorporated with Mh and Ta were expressed and purified with the yields as shown in **Figure S1 (A)**.

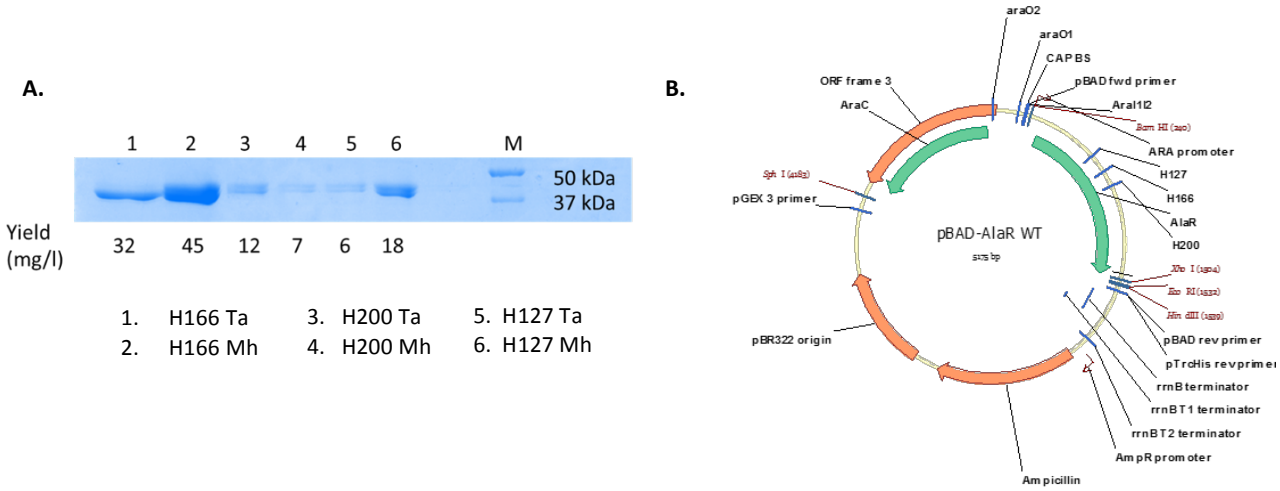


Figure S1. (**A**) Expression of alanine racemase mutants supplied with 2mM NCAA as detailed above. (**B**) pBAD-AlaRwt-His6x plasmid constructed for expression of WT and mutant enzymes.

6. Mass spectral data of alanine racemase H166 mutant enzyme:

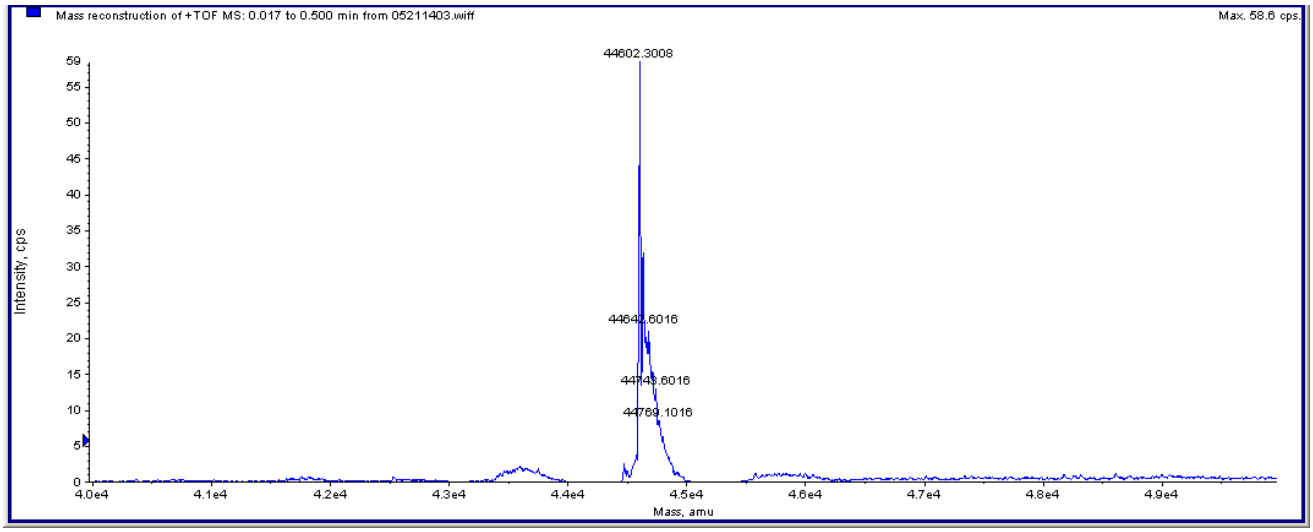


Figure S2. ESI-MS of alanine racemase H166-Mh mutant. Theoretical molecular weight is 44,601 Da which corresponds well with the major peak observed in the spectra of 44,602 Da.

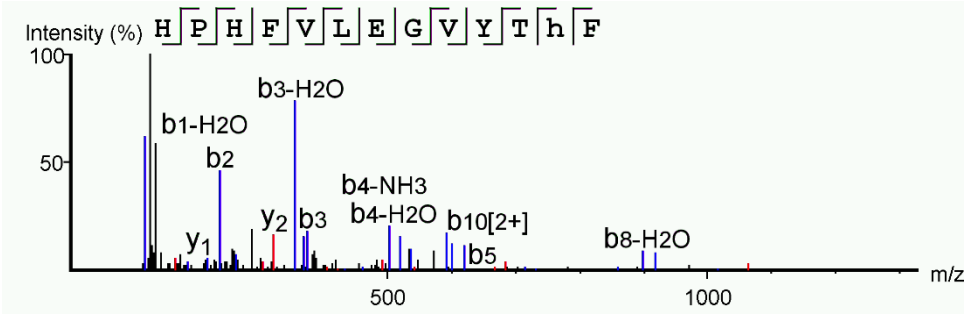
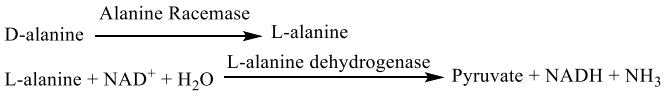


Figure S3. MS/MS analysis of alanine racemase H166-Ta mutant. The b1 ion indicates presence of thiazole alanine at the 166 position of the alanine racemase mutant sample.

7. Kinetic Assay for studying enzymatic activity:



The kinetic assay employed to study the enzymatic activity of WT alanine racemase and the mutant enzymes consisted of coupled reactions between conversion of D-alanine to L-alanine and subsequent reaction of L-alanine with L-alanine dehydrogenase to form NADH which is detectable by its absorbance at 340 nm. All the reactions were carried out at room temperature in a buffer containing 50 µM PLP (Pyridoxal Phosphate), 0.1 M KCl, 0.1 M CHES (N-Cyclohexyl-2-aminoethanesulfonic acid) Buffer (pH 9.0), 10 mM NAD^+, and 0.1 – 10 mM D-alanine and 2 units/ml of L-alanine dehydrogenase.[3] The extinction coefficient of NADH used for calculations was 6220 M^{-1} cm^{-1}. The enzyme concentrations were 0.11 nM for WT, 0.09 µM for H166-Ta, 0.16 µM for H166-Mh, 0.17 µM for H200-Ta, 0.12 µM for H200-Mh, 0.17 µM for H127-Ta and 0.37 µM for H127-Mh. All the measurements were made at 340 nm on UV-1800 UV-Spectrophotometer, Shimadzu®.

8. Circular Dichroism Analysis:

All the mutant protein samples were further purified on FPLC (Bio-Rad, NGC^{TM} Chromatography System) and dialyzed in 20 mM borate buffer (pH 9.1) to prepare their samples for measurement. Circular dichroism (CD) measurements were made on AVIV-62DS spectrometer using quartz cuvette of 1 mM pathlength. CD spectra was recorded at 25°C in 20 mM borate buffer (pH 9.1) purified by filtration before use. Data was collected from 190 nm to 280 nm in increments of 1 nm with averaging time of 10s. Concentrations of all protein samples were normalized to 0.022 mg/ml for helicity calculations. Fraction helicity was calculated using the ellipticity data at 222 nm obtained according to the method described by Rohl et al. (1997).[4] The % helicity of mutant samples as compared to WT alaR (22.7 %) were as follows: alaR H166Mh (22.9 %), alaR H166Ta (20.4 %), alaR H200Mh (21.6%), alaR H200Ta (19.9 %), alaR H127Mh (21.7 %) and alaR H127Ta (20.7 %).

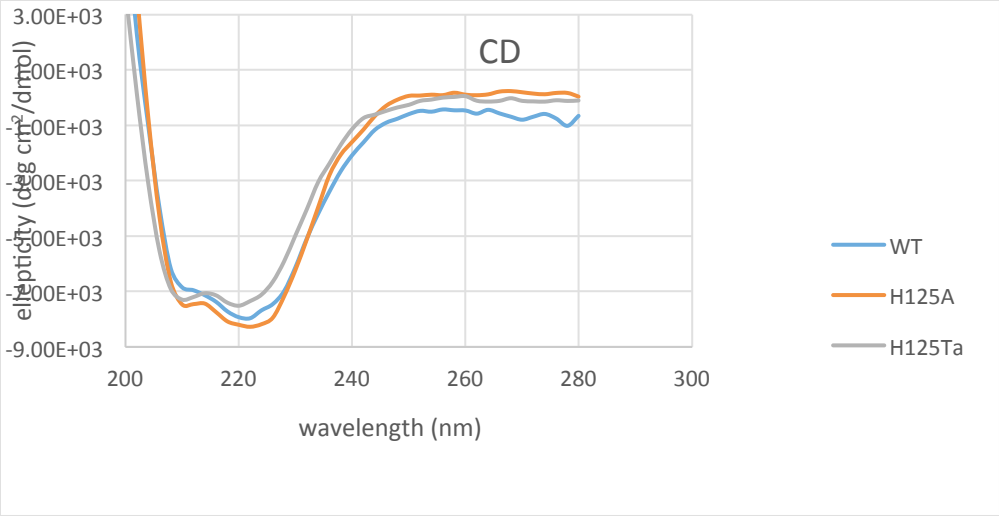


Figure S4. CD spectra of wild type, H125A, and H125Ta alanine racemases.

9. References:

[1] Y. S. Wang, X. Fang, A. L. Wallace, B. Wu, W. R. Liu, *J. Am. Chem. Soc.* **2012**, *134*, 2950-2953.
[2] J. T. Hammill, S. Miyake-Stoner, J. L. Hazen, J. C. Jackson, R. A. Mehl, *Nat. Protoc.* **2007**, *2*, 2601-2607.
[3] S. Sun, M. D. Toney, *Biochemistry* **1999**, *38*, 4058-4065.
[4] C. A. Rohl, R. L. Baldwin, *Biochemistry* **1997**, *36*, 8435-8442.